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ORIGINAL PAPER

Identities and distributions of the co-invading ectomycorrhizal fungal symbionts of exotic pines in the Hawaiian Islands

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Abstract Pine species have become invasive throughout the globe and threaten to replace native biota. The threat of pine invasion is particularly pressing in parts of the tropics where there are no native pines. The factors that govern pine invasion are not often well understood. However, key to pine survival is an obligate and mutualistic interaction with ectomycorrhizal fungi. Thus for pines to successfully invade new habitats compatible ectomycorrhizal fungi must already be present, or be co-introduced. The purpose of this study was to examine the community structure of non-native ectomycorrhizal fungi associated with pine invasions in the Hawaiian Islands. To accomplish this we executed a field and greenhouse study and used a molecular ecology approach to identify the fungi associating with invasive pines in Hawai'i. We show that: (1) ectomycorrhizal fungal species richness in non-native pine plantations is far less than what is found in pine's native range, (2) there was a significant decrease in average ectomycorrhizal fungal species richness as distance from pine plantations increased and, (3) *Suillus* species were the dominant fungi colonizing pines outside plantations. The keystone ectomycorrhizal fungal taxa responsible for pine establishment in Hawai'i are within genera commonly associated with pine invasions throughout the globe. We surmise that these fungi share functional traits such as the ability for long-distance dispersal from plantations and host tree colonization via spore that lead to their success when introduced to new habitats.

Keywords *Pinus* · Ectomycorrhizae · *Suillus* · Symbiosis · Plant–microbe interactions

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Introduction

Coniferous tree species, especially pines, are the most abundant and widespread invasive trees (Richardson and Rejmánek 2004). In fact, pines have become invasive in over 40 countries where they threaten to replace the native biota (Richardson and Rejmánek 2004). Previous research has shown that pine invasion

can produce dramatic effects on both above- and below-ground community structure, as well as alter abiotic ecosystem processes such as carbon sequestration and nutrient availability (Dickie et al. 2011; Dodet and Collet 2012). The threat of pine invasion is particularly pressing in parts of the southern hemisphere where there are no native pines. However, the factors that govern successful pine invasions are not often well understood (Richardson and Rejmánek 2004; Nuñez et al. 2009; Dickie et al. 2010; Nuñez and Medley 2011). One important biotic interaction for pine establishment and growth is their association with root inhabiting symbiotic fungi.

All pines have an obligate mutualism with symbiotic fungi known as ectomycorrhizal (EM) fungi (Read 1998). In return for supplying soil nutrients to their host trees, these fungi receive life-sustaining carbon from plant photosynthesis (Smith and Read 2008). EM fungi cannot establish and grow without their host trees; and in the absence of these fungi, pine survival is extremely limited (Read 1998; Thiet and Boerner 2007; Nuñez et al. 2009). Therefore, for pines to successfully invade new habitats, compatible EM fungal symbionts must already be present or be co-introduced. The interaction of non-native pines with EM fungi provides a clear example of one pathway or mechanism that can lead to pine invasions (Levine et al. 2003). However, determining the distributions and identities of EM fungi either already present, or introduced to new habitats can be challenging. These challenges include the inconsistent fruiting of many EM fungi, the inconspicuous aboveground fruit bodies of others, and the fact that aboveground EM community richness is often a poor predictor of which fungi are actively forming ectomycorrhizae with host trees (Gardes and Bruns 1996). However, the application of molecular based methods to the study of soil microbial ecology has led to substantial advances in understanding and cataloging the diversity of EM communities throughout the globe (Peay et al. 2008).

Despite the fact that alien pines are currently a major conservation concern, relatively few recent studies have used molecular techniques to determine the belowground community structure of the EM fungi that facilitate pine invasion (Collier and Bidartondo 2009; Nuñez et al. 2009; Dickie et al. 2010). Furthermore, tropical islands tend to be particularly vulnerable to biotic invasions (Vitousek et al. 1996). Although pines have frequently been introduced to the tropics, including islands, only one recent study in the Seychelles has used molecular methods to examine the EM fungi associated with non-native pines (Tedersoo et al. 2007). The purpose of the current study was to examine the extent of EM fungal introductions associated with non-native pines in the Hawaiian Islands and to use molecular techniques to determine their identities.

More native species have been eliminated and replaced by invasive organisms in Hawai‘i than anywhere else in the United States (Mooney and Drake 1986; Ikuma et al. 2002). Hawai‘i has no native pines and no known native EM fungi (Vellinga et al. 2009). However, starting in the early 20th century, extensive (>8,000 ha.) pine plantations were planted in Hawai‘i (Little and Skolmen 1989). Consequently, the establishment of these plantations in Hawai‘i represents not only a pine introduction into previously pine-free ecosystems, but likely an EM fungal introduction into previously EM-free soils (Koske et al. 1992). Recently, pines have escaped plantations and are expanding rapidly into native habitats in Hawai‘i. These events have raised concern about the consequences for native ecosystems, and how to stop further pine invasion (Oppenheimer 2002). Because significant positive feedbacks between non-native pines and non-native EM fungi are likely, determining the extent and function of these EM fungal introductions could be key in altering the processes of pine invasion.

The lack of native conifers and any recognized native EM fungi to Hawai’i makes these islands a model field system to examine the co-occurrence and feedbacks in the invasion process between two groups of unrelated, but obligately associated organisms. In this study, we examined the community structure of non-native EM fungi associated with pine invasions into previously EM fungi and pine-free habitats. We surveyed for EM fungi by sampling live pine roots in, and away from five pine plantations on two islands. Concurrently, by assaying soils for effective EM fungal inoculum from currently pine-free habitats, we aimed to assess the potential for future pine establishment at these sites. We predicted that overall EM community richness in the plantations would be lower than in pines’ native range and that escaped pines would be colonized with a subset of the EM fungi found within plantations.

Materials and methods

Field site descriptions

Field sites were located on the islands of Maui and Hawai'i. Site locations were chosen with the assistance of botanists and land managers from the Department of Interior's Haleakala National Park, Department of Forestry and Wildlife, and Department of Hawaiian Homelands. Aerial photographs were used to identify sites where pines were dispersing from plantations and establishing in previously pine-free habitats. Each pine plantation varied in size, age, and pine species (Table 1). In total, we chose five plantations from here on referred to as sites P1–P5. Previously pine-free habitats surrounding each plantation ranged from native subalpine shrublands and native bunchgrass vegetation in Haleakala National Park and Kula Forest Reserve on Maui, to habitats dominated by the native 'ōhi'a lehua trees (*Metrosiderous polymorpha*) and tree ferns (*Cibotium menziesii*) on Tree Planting Road, to non-native pastureland on Mauna Kea Access Road on the island of Hawai'i. All invaded habitats were dominated by either arbuscular or ericoid mycorrhizal host plant species (for details see Table 1). Site P2 had experienced a recent wildfire in 2007. The recent fire encouraged the spread of the fire-adapted closed-cone pine species *Pinus radiata* outside the original plantation's boundaries. At Site P3 close to the edge of the plantation the landscape was dominated by native and non-native trees, at about 500 m from the edge of the plantation the plant community shifted into a subalpine grassland dominated by endemic bunchgrass vegetation. Site P4 was located along the Mauna Kea Access Road, this was the smallest pine plantation included in this study (Table 1). The surrounding habitat was predominantly pastureland containing a mix of non-native annual grasses, and the biennial forb *Verbascum thapsus*. Site P5 was located on Tree Planting Road in the Kulani area on the island of Hawai'i. The vegetation closest to the plantation was dominated by small trees and woody shrubs and at a distance of about 200 m from the plantation's edge, the vegetation type shifted to dense stands of native tree ferns. In 1942, this site experienced a lava flow and therefore the soil was primarily lava rock with little to no organic layer.

Sampling scheme

We used the same sampling scheme at every site except at P5 where due to the rocky soil, we had to modify our sampling technique. Starting from the edge of each plantation, we walked in a straight line 1 km away from any surrounding plantations. We determined our distance from plantations' borders using a GPS (Garmin etrex, Olathe, KS, USA). To determine our distance from individual trees, we used a laser range finder (Nikon Forestry 550, Melville, NY, USA). Once we reached 1 km from plantations, we used sterilized trowels to collect approximately 250 ml of soil from three points 20 m apart to be used in our greenhouse bioassay experiment (see below). For each soil sample, the upper litter layer was removed and soil was sampled from the top 15 cm. This soil sampling scheme was repeated at 500, 100, 50, 10 and 1 m from the plantation border we originated from for all sites. Inside the plantations, 1 L of soil was collected from randomly selected spots. At site P2, we collected pinecones from the limbs *P. radiata* trees as seed sources for our bioassays.

In addition to the bulk soil samples, soil cores were collected from individual pine trees at four distance classes from each of the five plantations. We used a sterilized 10 cm long by 6 cm diameter metal soil corer to extract soil and roots. The furthest pine trees from each plantation were sampled first, these trees were located >250 m from the borders of plantations at an average of 615 m away. From each tree, three cores were collected from within 1 m of the base. In each of the remaining distance classes, 50–100, 10–50, and <10 m from plantations, two trees were each cored three times. Due to the recent volcanic origin of the soil in P5, we were unable to use our corer to sample pine roots. Instead, we used sterilized trowels and a pickaxe to dig out clusters of roots from three random points around the bases of individual pine trees. Ages and sizes of the trees varied from saplings a meter or less tall in P1, to mature trees 1.75–4.5 m tall in P2 and P3, to older and taller trees from 8 to 15 m tall in sites P4 and P5. The exact age of pines in the tropics is difficult to determine using standard methods such as increment coring due to the lack of growing season (Lanner 1966). Nevertheless, the pines sampled outside each plantation were younger than those within their respective plantations (Table 1). From an additional randomly selected six

Table 1 Site locations and descriptions of pine plantations in Hawaii from in this study

Plantation	Island	Location	Coordinates	Elevation (m)	Plantation age (years)	Size (hectares)	Dominant preexisting vegetation	Dominant pine species
P1	Maui	Haleakala national park	20°46′8.68″N 156°14′28.01″W	2084	63	320	*Styphelia tameiameiae*, *Vaccinium reticulatum*, *Sophora chrysophylla*, *Coprosma montana*, *Dodonaea viscosa*, *Dubautia menziesii*	*Pinus patula*/ *P. radiata*
P2	Maui	Kula forest reserve	20°41′13.24″N 156°18′46.66″W	2139	44	117	*Styphelia tameiameiae*, *Vaccinium reticulatum*, *Sophora chrysophylla*, *Coprosma montana*, *Dodonaea viscosa*, *Dubautia menziesii*	*P.pinaster*/ *P. radiata*
P3	Maui	Kula forest reserve	20°43′31.01″N 156°17′37.21″W	1921	44	61	*Metrosideros polymorpha*, *Acacia koa, Deschampsia nubigena*, *Pteridium aquilinum* var. *decompositum*	*P.pinaster*/ *P. radiata*
P4	Hawaii	Mauna Kea access road	19°42′57.70″N 155°26′44.82″W	2209	~50	3	Non-native grasses	*P.radiata*
P5	Hawaii	Tree planting road	19°37′42.93″N 155°14′45.49″W	1092	52	Unknown	*Metrosideros polymorpha*, *Vaccinium reticulatum Dicranopteris linearis, Cibotium menziesii*	*P. taeda*

trees inside each plantation, three soils cores per tree were also taken. In total, we extracted 38 soil cores from each site, for a grand total of 190 cores, including the root samples from P5. Bulk soil and soil cores were placed on ice for transportation to soil sorting stations.

Sampling of ectomycorrhizae from soil cores

From each soil core (or clump of roots for P5), eight random ectomycorrhizal (EM) root-tips were collected. After washing each core over a series of soil sieves, the smallest being 1 mm mesh, we placed the remaining pine roots on 10 cm diameter petri dishes with a 1 × 1 cm grid. Using a dissecting scope, we sampled eight EM roots closest to randomly-selected grid intersections. Each sample was frozen at −20 °C in 200 µl of 2X CTAB buffer (Gardes and Bruns 1993) for future molecular analysis. All soil cores were sampled for EM root-tips within 10 days of collection, and most within 2 days of collection.

Bioassays

Bulk soils and pinecones were transported on ice to the University of California Irvine where we set up a greenhouse bioassay experiment. Pinecones were heated at 80 °C to open the cones. Seeds were then extracted from the cones, de-winged, and surface sterilized in 500 ml of 30 % hydrogen peroxide and a few drops of Tween 20 for 20 min. Sterilized pine seeds were then repeatedly rinsed with deionized water and aseptically plated on petri dishes with moist filter paper. Seeds were allowed to germinate for 2 weeks before planting. Bioassay tubes were 115 ml volume cone-tainers (Stewe and Son Tangent, OR, USA). The drainage holes of each cone-tainer were

filled with poly-fill and a layer of sterilized sand. For each site we established four replicate bioassays representing each soil sample from points A, B, and C at each of the six distances from every plantation's border. For each distance class at each site, there were 12 bioassays, plus an additional 12 bioassays of soil from inside the plantations, for a grand total of 420 bioassays of live field soil. We also set-up eight additional control bioassays per site using gamma irradiated field soil collected 1,000 m from every plantation. In preparation for planting, each tube was filled with approximately 55 ml of soil. Soils from different sites and distances classes were spatially separated in racks to prevent any crossover contamination. Using sterile forceps, three seeds were placed in each bioassay cone-tainer. Seeds were then covered with a layer of sterilized sand. Bioassays were grown in the greenhouse under ambient temperatures and watered daily with deionized water. After 3 weeks in the greenhouse, bioassays were thinned to one seedling, and those that had no surviving seedlings were replanted. All bioassays were planted within 1 month of soil collection. After 7 months in the greenhouse, the bioassays were harvested. Each seedling was gently removed from its tube, and its roots were washed. The root systems of seedlings were sampled for EM root-tips in the same manner as the soil cores, except whole root systems were placed on the petri dish. EM roots were stored in 200 µl of 2X CTAB at −20 °C for future molecular analysis.

Molecular analysis of ectomycorrhizae

DNA of the EM root-tips sampled from the soil cores and bioassays was extracted using the Sigma Extract-n-Amp Plant kit (Sigma-Aldrich St Louis, MO, USA), following the manufacturer's guidelines with some slight modifications. Each root tip was placed in 20 µl of Extraction Solution, ground with sterile forceps and boiled, and then 60 µl of either the Sigma Dilution Solution or a 3 % BSA solution was added to every extraction. Extractions were stored at 4 °C. For each DNA extract, PCR was carried out in 25 µl total volume reactions using 2.5 µl of one-tenth concentrated DNA. The nuclear ribosomal internal transcribed spacer (nrITS) region was amplified with the fungal-specific primer combination ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and PCR conditions described in Gardes and Bruns (1993). PCR clean-up and single pass Sanger sequencing was performed by Beckman-Coultier Genomics (Danvers, MA, USA) using the primer ITS1F for sequencing.

In Sequencher v4.7, electropherograms of trimmed fungal sequences from all sites were manually checked for the quality of base pair calls, and those sequences that had low peak heights or multiple templates were removed. High quality sequences (quality score of >85 %) were binned at 95 % sequence similarity. From each bin, consensus sequences were created. To determine taxonomic affinities, consensus sequences along with singletons were compared to NCBI's GenBank database using the batch BLAST algorithm available from University of Alaska's Fungal Metagenomic Project (http://www.borealfungi.uaf.edu/). Generic or familial names from GenBank that matched our unknown sequences with at least 95 % Query Coverage and 95 % Max Identity were used to name the bins of our unknown sequences or singletons. If two consensus sequences shared the same best BLAST match (e.g., they matched the same sequence with similar coverage) they were given the same name with a unique taxon identification number (ex. Atheliaceae sp.1, Atheliaceae sp.2). For a few of our taxa, we had access to vouchered sequence databases and were able to name these at the specific level (T.D. Bruns pers. comm.). We determined the mycorrhizal status of the fungi identified in this study based on the records in Tedersoo et al. (2010). The dominant haplotype sequences from each bin, or singletons were submitted to GenBank (accession numbers JX898940-JX898978).

Data analysis

For our statistical analyses, only sequences of ectomycorrhizal fungi were included. To examine patterns of EM taxa abundance across sites and distance classes, we used the same calculations for both the soil core and the bioassay samples. At each distance class, we calculated the percent relative abundance of a taxon for all sites where it was present (Figs. 1, 2). Then, for the most abundant EM taxa, we used a general linear model to test if there were significant decreases or increases in these taxa relative abundances as distance from the plantations increased. Distance was log transformed to make the distribution of our data more symmetrical.

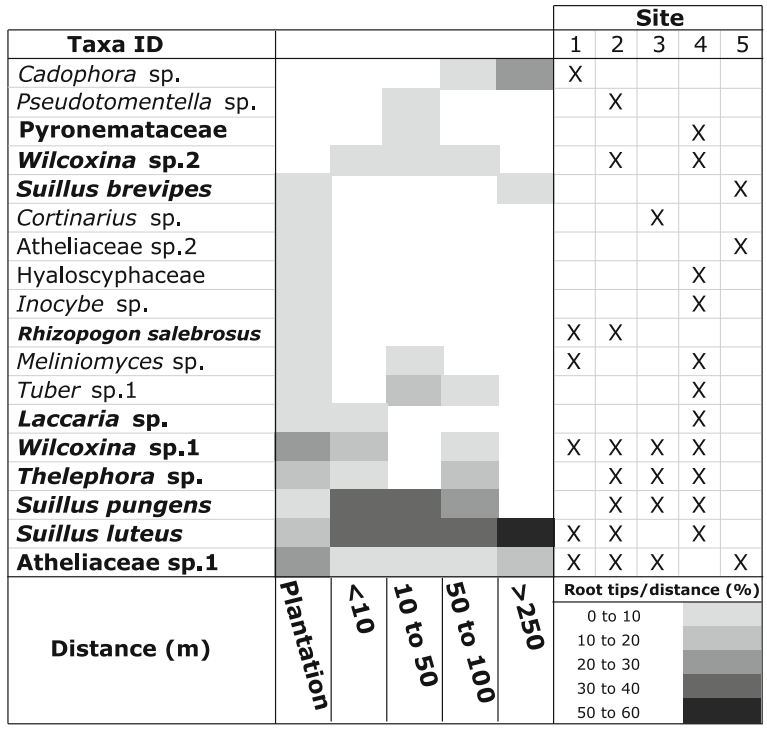

Fig. 1 Percent relative abundance of ectomycorrhizal fungi sampled from soil cores collected at various distances from five non-native pine plantations on the islands of Maui and Hawai'i. Taxa in *bold* are shared among soil cores and pine seedlings from an additional soil bioassay experiment (Fig. 2). Abundance was pooled across sites within each sampling distance. Presence/absence of each EM taxon at a particular site is indicated on the *right*

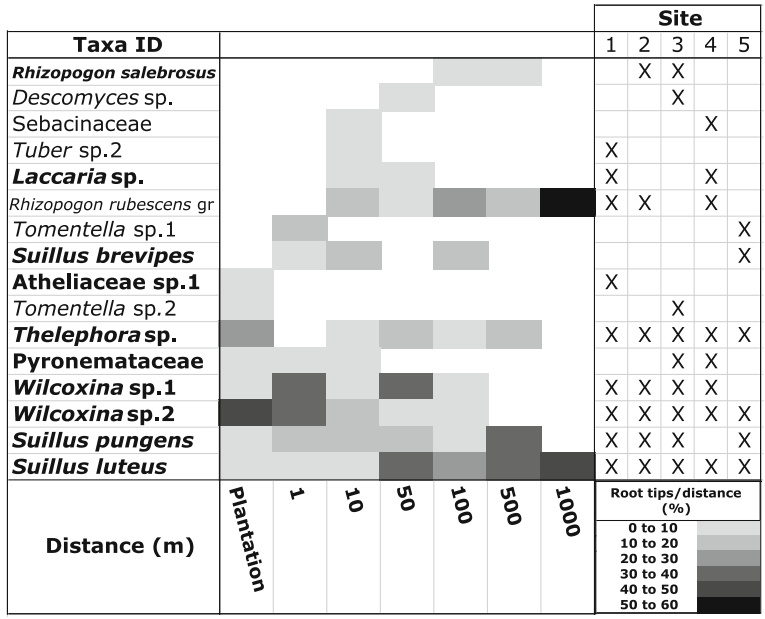

Fig. 2 Percent relative abundances of ectomycorrhizal fungi sampled from pine seedling bioassays grown in soils collected at various distances from five non-native pine plantations on the islands of Maui and Hawai'i. Taxa in *bold* are shared among seedlings and soil (Fig. 1). Abundance was pooled across sites within each sampling distance. Presence/absence of each EM taxon at a particular site is indicated on the *right*

To determine the relationship between overall richness of EM taxa as distance from plantations increased, we calculated the observed average number of taxa per a tree or bioassay at every distance and every site. Rarefaction of all taxa per site revealed that these are relatively species-poor communities (Supplementary Figure 1). To examine the relationship between EM richness and distance from plantations for the soil core samples, we used a general linear model with site as a random effect; the mean EM richness per tree for each site was regressed against the log transformed average of each distance class (i.e.,

0–10 m distance class equals 5 m average). For the bioassay samples, the same model was applied, but the regression was carried out on the mean EM richness per bioassay per site over the log of the sampling distance from the plantations. Because some replicate bioassays within a given point (A, B, or C) did not survive or generate high quality EM sequences, we pooled the data from the surviving bioassays within a distance class and site. Thus, our replication for the bioassays was by distance and site, rather than by sampling point, distance, and site. All of the above analysis were conducted with IBM SPSS v.20 (IBM Armonk, NY, USA).

To test for overlap of EM taxa present in the soil cores and bioassays we used the Ecosim (Entsminger 2012) range overlap model with species richness as a fixed factor and 50,000 randomizations. For the EM taxa shared between the two samples types and present at more than one site, we used the Ecosim species overlap model with species richness as a fixed factor and 50,000 randomizations to test for differences in the presence of these fungi among our sites (P1–P5). To compare the relative abundance of each EM taxon shared between soil cores and bioassays across similar distances independent of site, we used a one-way repeated measures ANOVA with "distance" as the independent variable and evaluated the interaction between soil core and bioassay abundance and distance for each taxon. All statistical tests were considered significant at $\alpha \leq 0.05$.

Results

Soil cores

Of the 190 soil cores taken, 23 contained no colonized pine roots. Of the 1,319 root-tips sampled, we had 59 % positive PCR amplification. Of these PCR products, 48 % were sequences identified as EM fungi. The remaining positive amplicons either did not meet our stringent quality score criteria or were identified as non-EM fungi. Total number of EM taxa from all sites was 18. Site P4 harbored the greatest number of EM fungi with 11 unique EM taxa; site P5 had the fewest with only three EM taxa detected (Fig. 1). The most common EM fungi across the majority of sites, both within and outside the plantations, were two species in the genus *Suillus*: *S. pungens* and *S. luteus* (Fig. 1). The relative abundance of *S. luteus* increased significantly as distance from plantations increased ($P = 0.023$). Additionally, a taxon in the family Atheliaceae was detected in all sites except P4 both within and outside plantations, but was far less abundant than the two species of *Suillus*, and its relative abundance did not change significantly over distance from the plantations ($P = 0.441$, Fig. 1). EM fungi in the genera *Wilcoxina* and *Thelephora* were also common amongst almost all sites (except P5), within and up to 100 m from the edge of plantations (Fig. 1). Other EM fungi in the genera or families *Cadophora*, *Pseudotomentella*, *Cortinarius*, *Inocybe*, *Tuber*, *Laccaria,* Pyronemataceae, Hyaloscyphaceae and another species of *Suillus*, *S. brevipes*, were site-specific and (except for *Cadophora*) tended to be rare regardless of location (Fig. 1).

The most common non-EM fungi were root endophytes of unknown function, such as *Phialocephala* sp., *Ceratobasidum* sp. and *Phialophora* sp. At site P5, we also detected two fungi inside plantations to over 250 m away belonging to the order Helotiales. Both taxa were close BLAST matches to ericoid mycorrhizal fungi in the *Hymenoscyphus ericae* complex. However, neither were close matches to *Meliniomyces* spp., which are the only fungi within the *H. ericae* complex thought to form functionally significant EM associations (Grelet et al. 2009; Vralstad et al. 2000). In addition, we found five Basidiomycetes including a *Chroogomphus* sp. and a taxon in the Auriculariales, and one Asocomycetes of undetermined mycorrhizal status, which were not included in our analyses.

As distance from pine plantations increased, there was a significant decrease in EM richness per tree; this relationship was fairly strong across all sites ($R^2 = 0.16$, $P = 0.011$). The observed average number of EM taxa per tree inside plantations was 1.7 (range 1.14–2.06). At distances of greater than 250 m from the edge of plantations the observed average number of EM taxa per tree was 1.13 (range 1–1.33). Plantation age and size were not significant predictors of overall EM richness.

Bioassays

Unfortunately, not all of the pine bioassays survived, and some seedlings that did survive had no EM colonization (Supplemental Table 1). Seedling survivorship was significantly different between distances from plantations ($F = 3.88$, $P = 0.006$), but not

between sites ($F = 0.461$, $P = 0.764$). However, based on a linear regression model there was not a significant correlation between distance from the plantation and seedling survivorship ($R^2 = 0.001$, $P = 0.892$). In total, of the 420 bioassays that we planted, 172 survived, from which we sampled a total of 1,371 root-tips. From the total root-tips sampled, we had an overall total of 46 % positive PCR amplification. This overall low PCR success rate may be partially explained by our bioassay sampling strategy. Because some species of EM fungi do not form clearly visible hyphal sheaths or mantles, to avoid discarding these cryptic colonizers we called root-tips mycorrhizal if they lacked root hairs. If PCR success rates are broken-down by distance class, we had greater PCR success from bioassay seedlings grown in soils collected inside, or 1 m from the plantations (62 and 66 % success rates respectively), versus bioassays from soils collected 500 m to 1 km outside plantations (26 and 21 % success rates respectively). Thus, based on our decreased PCR success rates as distance from the plantations increased, we infer that bioassays from soils at distances greater than 500 m from plantations had substantially less frequent EM colonization than those from soils collected inside plantations. Of the total positive amplicons sequenced, 69 % were identified as EM fungi, and these root-tips were from 107 bioassays (Supplementary table 1). From the bioassays we detected a total of 16 EM taxa from all sites within and outside plantations. Unlike the field sampling, on the bioassayed seedlings we did not detect a significant correlation between average EM OTU richness and distance from plantations ($R^2 = 0.003$ $P = 0.195$).

In the bioassays, *Suillus luteus* was detected at all five sites. As in the soil cores, *S. luteus* increased in relative abundance as distance from plantations increased ($P = 0.001$, Fig. 2). Also common at all five sites from soils collected less than 100 m from plantations were two taxa in the genus *Wilcoxina* and a *Thelephora* species. *Wilcoxina sp.2* tended to be more abundant inside and within 50 m of the edges of plantations than further afield and had a significant decrease in relative abundance as distance from plantations increased ($P = 0.032$, Fig. 2). We found a suite of EM taxa only in soils collected at distances 1 m or greater from plantations' edges. For example, *Rhizopogon salebrosus* was found at distances greater than 100 m and less than 500 m from the borders of two plantations (Fig. 2). Another species of *Rhizopogon* (in the *R. rubescens* group) was found at three sites at distances of 10 to 1,000 m from plantations. Along with *S. luteus*, this was the most abundant species detected at 1,000 m (Fig. 2). Also, *S. brevipes* and a *Tomentella* species were found in soils collected 1 m from site P5, but not inside plantations. Additional EM taxa in the genera *Laccaria*, *Tuber*, *Descomyces* and the order Sebacinales were found only in bioassay soils from distances greater than 10 m outside plantations (Fig. 2). Inside the plantations seedlings were dominantly colonized by species of *Thelephora* and *Wilcoxina*, Atheliaceae sp.1 and *Tomentella* sp.2, which did not colonize bioassays grown in soils collected outside the plantations.

We also detected one unknown Basidiomycete in four bioassays from site P3. Because this fungus' best BLAST match was to an "unknown ectomycorrhizal species" it was not included in our statistical analyses. Nor did we include three Ascomycete taxa of unknown function found in one bioassay from P2, three from P4, and two from P5. In addition, bioassays from sites P5 and P3 in soils from 50 to 100 m away from the plantations had two fungal taxa that were close matches to the *H. ericae* complex. However, as with the soil core samples, these sequences were not close matches to *Meliniomyces* spp. Thus, these fungi were not included in our statistical analyses. All control bioassays were uncolonized except for those from site P4, which were colonized by a species of *Hebeloma*. This species was also detected in six bioassays from site P2 in soil collected 1,000 m away from the plantation. Due to the potential of *Hebeloma* being a greenhouse contaminant, these bioassays were not included in the statistical analyses.

Differences between the EM communities in the soil cores and bioassays were not significant ($P = 0.06$), with 42 % of the total taxa shared between the sample types, 33 % of taxa specific to the soil cores, and 25 % specific to the bioassays (Figs. 1, 2). Of the shared EM taxa between sample types, their presence among sites was not significantly different from a community where species composition was assigned at random ($P = 0.6$). *Suillus luteus*, *S. pungens*, and *Wilcoxina* sp.1 were found in both sample types, multiple sites, and they had similar relative abundances at similar distances in both sample types ($P = 0.509$, 0.847, 0.497 *S. luteus*, *S. pungens*, and *Wilcoxina* sp.1 respectively). Of these three taxa,

only *S. luteus* was found at distances greater than 500 m from plantations in the bioassays, and greater than 250 m from plantations in the soil cores (Figs. 1, 2). *Wilcoxina* sp.1, which was also detected in both sample types, was relatively more abundant in bioassays at distances greater than 10 m from plantations than in the soil cores ($P = 0.026$, Figs. 1, 2). Some other taxa, shared between the sample types such as *Laccaria* sp. and *R. salebrosus* were only detected outside plantations from bioassays, but were found inside plantations in the soil core samples (Figs. 1, 2). *Thelephora* sp. had a similarly patchy distribution in both bioassays and soil cores ($P = 0.915$), but was not found at distances greater than 500 m from plantations in the bioassays or greater than 100 m in the soil cores (Figs. 1, 2).

Discussion

Overall EM richness from non-native pine plantations in Hawai'i was substantially lower than EM communities in native pine forests, which can contain over a 100 species in a 0.5 hectare area (Allen et al. 1995). This decrease in EM richness is common in other areas throughout the globe where pines and EM fungi have been introduced (Dunstan et al. 1998; Orlovich and Cairney 2004; Nuñez et al. 2009; Dickie et al. 2010; Walbert et al. 2010). Our results, in combination with other studies, indicate that a restricted suite of EM fungi are the common dominants with pine introductions. These fungi are a subset of the ones associated with pines in their native ranges, but their relative abundance varies depending on habitat (native versus non-native) (Baar et al. 1999; Taylor and Bruns 1999; Izzo et al. 2006; Hoeksema et al. 2012). The EM fungi commonly found associated with pine introductions include species in the genera *Suillus* (Figs. 1, 2, Dunstan et al. 1998; Giachini et al. 2000; Orlovich and Cairney 2004; Nuñez et al. 2009; Vellinga et al. 2009; Dickie et al. 2010; Walbert et al. 2010), *Thelephora* (Figs. 1, 2, Dunstan et al. 1998; Giachini et al. 2000; Orlovich and Cairney 2004; Walbert et al. 2010), *Rhizopogon* (Figs. 1, 2, Giachini et al. 2000; Orlovich and Cairney 2004; Tedersoo et al. 2007; Nouhra et al. 2008; Nuñez et al. 2009; Walbert et al. 2010), *Wilcoxina* (Figs. 1, 2, Nuñez et al. 2009), and fungi in the family Atheliaceae (Figs. 1, 2, Dickie et al. 2010). Other EM taxa such as those in the genera *Inocybe*, *Laccaria*, *Tuber*, *Tomentella* and *Pseudotomentella* are also commonly associated with non-native pines, but tend not to be highly abundant fruiters or root colonizers (Figs. 1, 2, Dunstan et al. 1998; Giachini et al. 2000; Orlovich and Cairney 2004; Vellinga et al. 2009; Dickie et al. 2010; Walbert et al. 2010). Interestingly, the dominant EM fungi associated with pine introductions in Hawai'i and elsewhere, are those that readily colonize hosts by spore (Taylor and Bruns 1999; Bruns et al. 2009; Ishida et al. 2008; Nara 2009). These include species in the genera *Suillus*, *Rhizopogon*, *Inocybe*, *Tuber*, *Tomentella*, *Wilcoxina* and *Laccaria* (Mikola 1988; Baar et al. 1999; Taylor and Bruns 1999; Izzo et al. 2006; Nara 2009). Many EM Species with a spore colonization strategy are common in early forest succession and thought to be ruderal species that can rapidly exploit newly available resources such as host tree roots or soil nutrients (Deacon and Fleming 1992). Conversely, in mature native pine forests the dominant EM fungi include species that primarily colonize their host trees via vegetative growth rather than spores (Taylor and Bruns 1999). Except for a handful of species (ex. *Cortinarius* sp. and *Descomyces* sp.) these fungi were absent in our system. Because both bioassay seedlings and in situ pine roots dominantly harbored species with a spore colonization strategy, the differences in EM community composition between our two sample types were small compared to what might be expected in native pine habitats.

Little is known about which functional traits are important for an EM fungus to successfully establish in a new habitat and abet their host plants. Key traits of successful non-native or native pioneer EM fungi have been reported to include the ability for relatively long-distance dispersal across a given landscape (Nuñez et al. 2009; Vellinga et al. 2009; Peay et al. 2010, 2012) and colonization of host trees by spore (Deacon and Fleming 1992; Ishida et al. 2008; Collier and Bidartondo 2009). The current study provides additional support for the importance of these factors. From our bioassay experiment, we found that *S. luteus,* the most common EM fungus in this study, could disperse spores at least 1,000 m from the borders of pine plantations and at least 500 m from single pine trees outside plantations (Fig. 1). Also, this species readily colonized seedling bioassays (Fig. 2), which tend to select for EM fungi with a spore colonization strategy (Taylor and Bruns 1999; Bruns et al. 2009;

Ishida et al. 2008; Nara 2009). Our findings are congruent with other recent studies of the spore dispersal abilities and colonization strategies of *Suillus* species. Nuñez et al. (2009) found that *S. luteus* was present in soils collected over 1,000 m from non-native pine plantations in Argentina. Similarly, Peay et al. (2012) found that *Suillus pungens* was the only species capable of consistently colonizing pine seedlings at distances greater than 10 km from native pine forest boundaries. Overall, we found no evidence of dispersal limitation in our bioassayed EM fungal communities at distances up to 1 km.

Additional factors that may be important for the successful establishment of EM fungi in Hawai'i and elsewhere, are positive interactions among non-native organisms. For example, in our study two *Rhizopogon* species, and two *Tuber* species colonized bioassays and in situ roots. Both these genera dominantly rely on mammals for dispersal across landscapes (Izzo et al. 2005). Because there are no native ground mammals to Hawai'i, the most likely culprits for *Rhizopogon* and *Tuber* dispersal are non-native boars, deer, and rats (Mooney and Drake 1986). *Rhizopogon* species are also host specific symbionts to species in Pinacaeae. These positive interactions between non-native plants, fungi, and mammals provides a compelling example an invasion meltdown where unrelated organisms facilitate each other's rates of establishment and degree of impact (Simberloff 2006). In Hawai'i the direct negative impacts of non-native mammals on native ecosystems is well documented (Mooney and Drake 1986). However, to the best of our knowledge this study is the first, albeit indirect example of how non-native mammals are positively influencing the success of non-native symbiotic fungi and in-turn plant invasions.

The most successful non-native EM fungi likely have a combination of traits that lead to their overall success and dominance when introduced to new habitats. For example, *Suillus* species can form relatively long-lived spore banks (Nguyen et al. 2012), disperse long distances (this study and Nuñez et al. 2009; Peay et al. 2010, 2012), and are commonly eaten and dispersed by mammals (Ashkannejhad and Horton 2006). This pattern is exemplified by the significant increase in *S. luteus*' relative abundance as distance from plantations increased in both the soil core and bioassay samples from this study. It should be noted that we removed a portion (approximately 10 %) of the *Suillus* sequences from our dataset because they did not meet our strict quality score criteria. This was mainly due to the presence of multiple peaks in the electropherograms of raw sequences, indicating that *Suillus* may have multiple ITS copies due to relaxed concerted evolution (Ian Dickie pers. comm.). Thus our data likely represents an underestimation of the relative abundance of *Suillus* species in our study system.

Among the EM fungi found only within plantations, a lack of successful traits for invading new habitats may explain their absence outside plantations; and in turn the significant decrease in EM species richness colonizing in situ roots as distance from plantations increased (Figs. 1, 2). From a theoretical standpoint, if the pine plantations are considered EM fungi host "mainlands", and escaped pine trees are "islands", decreasing species richness with increasing distance from these mainlands is consistent with other studies of island biogeography (MacArthur and Wilson 1967; Cadotte 2006; Peay et al. 2010). Though we did not explicitly test the role of island size on predicting species richness, if we consider each of our pine plantations, rather than individual trees, as islands we found no significant relationship between plantation size and EM richness. This finding is contrary to those of Peay et al. (2007), who reported strong species-area relationships for EM fungi associated with native pine host tree islands. The discrepancy between our two studies is likely due to the human transport of EM inocula to the pine plantations of Hawai'i. However, our finding that approximately 16 % of the change in EM OTU richness could be explained by isolation from pine plantations is similar to that of another study by Peay et al. (2010), and studies of plant species richness on oceanic islands summarized by Cody (2006). Other biotic and abiotic factors such as interactions with native soil microbes (Klironomos 2002; Mitchell et al. 2006; Kohout et al. 2011) and niche partitioning of soil resources (Chapela et al. 2001; Funk and Vitousek 2007; MacDougall et al. 2009) may also be important in determining the distribution of EM communities across previously EM-free habitats, and they deserve further study.

We found that independent of distance from plantations, pine roots collected in situ from mature trees were colonized by EM fungi. Conversely, from our bioassays, as distance from plantations increased, we found increased rates of PCR failure, which we

infer as an increase in the number of uncolonized seedlings. In total, only two of our five sites had substantial EM colonization of bioassay seedlings grown in soils collected 1,000 m from plantations. Consequently, pines may survive in the absence of EM fungi for at least a portion of their early stages of development before becoming colonized (Collier and Bidartondo 2009). Thus, early pine establishment may take place in the absence of EM fungi, but long-term survival depends upon forming symbioses with EM fungi. Furthermore, EM fungi, especially species in the genus *Suillus*, can be prolific spore producers (Peay et al. 2012) with survival rates of over 6 years (Nguyen et al. 2012), and it takes relatively few spores to colonize new hosts (Bruns et al. 2009). Therefore, we predict that many non-native EM fungi in Hawai'i will fairly rapidly overcome any restrictions that a lack of propagule pressure may impose on their distributions and their host plants' establishment.

Plant and fungal invasions pose a serious threat to the integrity of native ecosystems, especially the sensitive habitats of the Hawaiian Islands. Left unchecked, pine invasions have been shown to have significant negative effects on native plant species richness, soil microbial communities and soil nutrient availability (Dickie et al. 2011). The costs of altering these ecosystem properties by pine invasions can sometimes be reconciled by the economic gains from timer harvest (Dodet and Collet 2012). However, in Hawai'i, the quality of the lumber produced from the exotic pines is too poor to make timber operations viable, or to justify the potential negative impacts of the plantations on ecosystem services such as fresh water availability (Kagawa et al. 2009). The results of this study add new information to the currently small body of research regarding plant and EM fungal invasions worldwide. Because *Suillus* species are common associates of pine invasions in Hawai'i and around the globe, we recommend that they not purposefully be transported as inoculum to aid in the establishment of new plantations.

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